

# Relationships of fibre, lignin, and phenolics to in vitro fibre digestibility in three perennial grasses

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## Abstract

Livestock performance can be improved by increasing the digestibility of feeds, one of the key elements of nutritional quality. Digestibility of feeds can be improved by breeding forage crops with modified cell-wall structure, increasing the potential availability of energy from the cell wall to rumen microbes and livestock. The objectives of this research were to identify interrelationships among lignin and phenolic components of the fibre fraction of three perennial grasses and to determine their influence on in vitro fibre digestibility. Differences in etherified and esterified ferulate and esterified *p*-coumarate among clones of three perennial grasses were generally repeatable across harvests. The concentration of neutral detergent fibre (NDF) and Klason lignin within the NDF fraction were the factors most limiting to 24-h in vitro digestibility, with NDF being the most important. Klason lignin and etherified ferulate were the factors most limiting to 96-h in vitro digestibility for all three species. Due to its positive correlation with NDF, selection for low etherified ferulate should be avoided in smooth brome (*Bromus inermis* Leyss) and cocksfoot (*Dactylis glomerata* L.). It should be possible to select and breed for low concentrations of lignin to improve digestibility without decreasing NDF in these two species. However, in reed canarygrass (*Phalaris arundinacea* L.), both lignin and etherified ferulate were positively correlated with NDF, indicating that selection for increased

**Abbreviations:** DM, dry matter; IVNDFD, in vitro neutral detergent fibre digestibility; NDF, neutral detergent fibre; NIRS, near infrared reflectance spectroscopy; PCA, *p*-coumarate; SEP, standard error of prediction

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digestibility should be based directly on some measure of in vitro digestibility to avoid the fitness problems associated with reduced NDF.

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**Keywords:** Smooth brome grass; Cocksfoot; Reed canarygrass; Lignin; Ferulate; *p*-Coumarate

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## 1. Introduction

Livestock performance can be improved by increasing the digestibility of feeds, one of the key elements of feed quality. Digestibility of feeds can be improved by breeding forage crops with modified cell-wall structure, increasing the relative availability of energy in the cell wall to rumen microbes and livestock (Casler, 2001; Fahey and Hussein, 1999). Because effective plant breeding requires evaluation of thousands of forage samples within a relatively short time period and under reasonably uniform conditions, in vivo digestibility cannot be used as a direct selection criterion in plant breeding programs. Indirect measures of digestibility are required for the selection and breeding process, followed by validation using in vivo measurements of digestion, lactation, weight gain or other indicators of animal performance (Argillier et al., 1998; Casler, 2001).

In vitro dry matter digestibility (IVDMD) has received considerable attention from forage breeders, with the demonstration of heritable variation in numerous species (Buxton and Casler, 1993). Genetic improvements in IVDMD are generally a result of decreases in cell-wall concentration, decreases in lignin concentration, or increases in water-soluble carbohydrate concentration (Casler, 2001). In vitro neutral detergent fibre digestibility (IVNDFD) has received relatively little attention from forage breeders, despite its potentially greater relevance to cell-wall utilization by ruminants. Because the cell wall represents the most indigestible portion of a forage plant, the digestibility and composition of the cell wall may comprise the factors most limiting to livestock production on high-forage diets (Van Soest, 1994).

Within grass cell walls, the relative abundance of lignin and the frequency of ferulate cross-links with arabinoxylans appear to be the most important factors limiting energy utilization by rumen microbes (Casler, 2001; Jung and Deetz, 1993). In vitro neutral detergent fibre digestibility can be increased by reducing lignin concentration or cross-linking between lignin and cell-wall carbohydrates (Casler, 2001). Lignin concentration has been identified as a key factor limiting IVNDFD in lucerne (*Medicago sativa* L.) (Jung and Lamb, 2003) and smooth brome grass (*Bromus inermis* Leyss) (Casler and Jung, 1999; Jung and Casler, 1991). The concentration of etherified ferulate, a measure of cross-linking between lignin and arabinoxylans, has a strong negative influence on IVNDFD of smooth brome grass, an effect that seems to be independent of the concentration of lignin in the fibre fraction (Casler and Jung, 1999). The objectives of this research were to identify interrelationships among lignin and phenolic components of the fibre fraction of three perennial grasses and to determine their influence on IVNDFD.

## 2. Materials and methods

Selection nurseries of orchard grass (*Dactylis glomerata* L.) and reed canarygrass (*Phalaris arundinacea* L.) were established from 70-day-old plants in May 1997 and a similar nursery for smooth brome grass was established in 1998. Each plant was raised in a glasshouse from an individual seed. For cocksfoot, the selection nursery consisted of a total of 1160 plants, 40 plants from each of 29 cultivars and experimental populations, all classified as medium-late to late in reproductive maturity. For reed canarygrass, the selection nursery consisted of a total of 2000 plants, 40 plants each from 50 cultivars and experimental populations, all classified as low in gramine and free of tryptamine and  $\beta$ -carboline alkaloids. The experimental design for both cocksfoot and reed canarygrass nurseries was a randomized complete block with four replicates and each plot consisting of a row of 10 plants from a population. For smooth brome grass, the selection nursery consisted of 960 plants from population WB19e, a broad-based population with considerable genetic variation for lignin, ferulates, and in vitro digestibility (Casler and Jung, 1999). All plants were planted on a 0.9 m spacing. Plants were fertilized with  $56 \text{ kg N ha}^{-1}$  prior to each growth cycle and weeds were controlled by a combination of hand weeding and pre-emergence herbicides (Falkner and Casler, 1998).

For each nursery, all plants were harvested in early spring and mid-summer of the first 2 years following the establishment year—1998/1999 for cocksfoot and reed canarygrass and 1999/2000 for smooth brome grass. The first harvest occurred when plant canopies were approximately 20 cm tall in spring. Following this harvest, plants were allowed to grow until anthesis, when they were cut and allowed to regrow. The second harvest occurred when the average plant canopy was again approximately 20 cm tall, generally in mid- to late July. Plants were fertilized with  $90 \text{ kg N ha}^{-1}$  in early spring and at the initiation of their regrowth cycle in each year. Plants were harvested by hand at a cutting height of 9 cm. All harvested plant samples consisted entirely of leaf blades and sheaths to avoid confounding results with leaf–stem ratio differences (Casler, 1999a,b). Only those plants with acceptable vigor, as determined by canopy height similar to the target of 20 cm, were sampled at any given harvest date. Samples were stored in paper bags and dried at  $60^\circ\text{C}$ .

Following the second harvest of the 2nd year for each nursery, plant samples were retained only for those plants represented in all four harvests. This resulted in population sizes of 280 cocksfoot plants, 729 reed canarygrass plants, and 775 smooth brome grass plants. Dry samples were ground through a 1 mm screen of a Wiley-type mill and scanned on a near-infrared reflectance spectrophotometer (NIRS). Calibration sample subsets were chosen by the NIRS software using cluster analysis of reflectance spectra (Shenk and Westerhaus, 1991). The calibration subsets consisted of 78 samples of smooth brome grass, 70 samples of cocksfoot, and 60 samples of reed canarygrass. Neutral detergent fibre (NDF) concentration was determined using the procedure of Van Soest et al. (1991), omitting the sodium sulfite,  $\alpha$ -amylase, and ash-correction steps. Klason lignin concentration was measured as the ash-free residue remaining after cell-wall polysaccharide hydrolysis (Theander et al., 1995). The Klason lignin procedure was utilized to remain consistent with our previous work on selection and breeding for lignin

Table 1

Calibration statistics for near-infrared reflectance spectroscopy analysis of 78 smooth bromegrass clones, 70 cocksfoot clones, and 60 reed canarygrass clones<sup>a</sup>

Variable (g kg <sup>-1</sup> DM) <sup>b</sup>	Smooth bromegrass			Cocksfoot			Reed canarygrass		
	Mean	SEP	R <sup>2</sup>	Mean	SEP	R <sup>2</sup>	Mean	SEP	R <sup>2</sup>
NDF	548	20	0.92	608	23	0.89	600	19	0.93
Klason lignin	87	10	0.46	110	9	0.73	95	12	0.67
Esterified ferulate	2.55	0.46	0.36	2.66	0.32	0.54	3.64	0.57	0.51
Etherified ferulate	2.76	0.63	0.60	1.67	0.44	0.78	1.59	0.31	0.63
Esterified PCA	1.45	0.82	0.52	1.49	0.51	0.60	2.44	0.45	0.59
24-h NDF residue <sup>c</sup>	241	30	44	224	38	42	227	25	65
96-h NDF residue <sup>c</sup>	120	19	71	118	20	83	114	16	77

<sup>a</sup> SEP, standard error of prediction.

<sup>b</sup> NDF, neutral detergent fibre; PCA, *p*-coumarate; DNDF, in vitro digestible NDF; DM, dry matter.

<sup>c</sup> Indigestible residue remaining after designated rumen-fluid incubation time, neutral-detergent refluxing, and filtering (g kg<sup>-1</sup> DM).

and etherified ferulate (Casler and Jung, 1999; Jung and Casler, 1991) and because it provides an estimate of all functional lignin in the cell wall (Hatfield et al., 1994; Jung et al., 1999; Lowry et al., 1994). Esterified ferulate and *p*-coumarate (PCA) concentrations in the cell wall were determined by 2 M NaOH extraction and HPLC analysis (Jung and Shalita-Jones, 1990). Concentration of etherified ferulate was computed as the difference between total ferulate, obtained by 4 M NaOH extraction at 170 °C for 2 h, and the esterified fraction (Iiyama et al., 1990). Lignin and phenolic acids were determined on duplicate samples. In vitro digestible NDF was determined in triplicate using 24- and 96-h fermentations according to the procedure of Casler (1987). Fermentation times of 24 and 96 h were used to provide estimates of rapidly and potentially digestible NDF fractions, respectively. Means over laboratory replicates were used to calibrate the NIRS separately for each of the three species with calibration statistics shown in Table 1. All variables were converted from a dry-matter basis to an NDF basis after predictions were generated using NIRS. In vitro digestibility of the NDF fraction was computed from the indigestible NDF residue and total NDF as described by Casler (1987).

All data were analyzed by analysis of variance with harvests as a fixed effect, clones as a random effect, and the clone × harvest interaction as the error term. Repeatability across harvest dates was computed as the intraclass correlation coefficient using the formula  $r_1 = s_G^2 / (s_G^2 + s_{GH}^2)$ , where  $s_G^2$  is the estimated variance component for clones and  $s_{GH}^2$  is the estimated clone × harvest interaction. The intraclass correlation is zero when repeatability across harvests is nil and unity when repeatability is perfect. Pearson correlation coefficients were used to estimate the inter-relationships among NDF, lignin, and phenolic variables. The standardized partial least squares method (Draper and Smith, 1981) was used to estimate the standardized and independent effects of NDF, Klason lignin, and phenolic variables on 24- and 96-h IVNDFD.

### 3. Results

Variability among clones was repeatable across harvests for all variables measured on all three species ( $P < 0.001$  in all cases, except for 24-h IVNDFD of reed canarygrass). Intraclass correlation coefficients were lowest for 24-h IVNDFD for all three species (Table 2), indicating the difficulty in precisely measuring clonal differences for digestibility following relatively short digestion periods. Repeatability tended to be greatest for cocksfoot, followed by smooth brome grass and reed canarygrass.

Lignin and phenolic acid profiles of leaf tissue differed among the three grass species (Table 3). Smooth brome grass had the lowest Klason lignin concentration and relatively similar amounts of etherified and esterified ferulates. Cocksfoot had the highest concentration of Klason lignin in the fibre fraction, while both cocksfoot and reed canarygrass had more esterified ferulate than etherified ferulate. Differences among species in total fibre, measured as NDF, were parallel to differences in lignin concentration. *p*-Coumarate ester profiles were consistent across the three species. Variability among clones for phenolic acids did not vary among species, with the single exception of relatively high variability for esterified *p*-coumarate of reed canarygrass. Cocksfoot showed the greatest variability for IVNDFD, despite having the smallest population size.

Standardized partial least squares regression allows estimation of the direct and independent effects of each cell-wall or fibre component on IVNDFD. Because Klason lignin and ferulate components showed the only evidence for significance in the full model, the *p*-coumarate ester component was removed and the model was recomputed (Table 4). Lignin and NDF were the only two consistently important factors influencing 24-h IVNDFD, both with negative effects. The concentration of NDF was two to four times more important than Klason lignin in its effects on 24-h IVNDFD. Etherified ferulate had a significant negative effect on 24-h IVNDFD of cocksfoot and reed canarygrass, an effect that was similar in magnitude to the effects of Klason lignin. Residuals were relatively large for 24-h IVNDFD, indicating that there was a relatively large amount of variability that was not explained by these factors.

In contrast, 96-h IVNDFD was influenced by Klason lignin and etherified ferulate within all three species (Table 4). These effects were similar in magnitude for smooth brome grass, but the effect of Klason lignin was almost twice as great as the effect of etherified ferulate

Table 2  
Intraclass correlation coefficients for clones of three perennial grasses evaluated over four harvest dates<sup>a</sup>

Variable <sup>b</sup>	Smooth brome grass	Cocksfoot	Reed canarygrass
NDF	0.40	0.47	0.29
Klason lignin	0.41	0.67	0.41
Esterified ferulate	0.58	0.63	0.37
Etherified ferulate	0.44	0.51	0.33
Esterified PCA	0.41	0.52	0.34
24-h IVNDFD	0.31	0.36	0.08
96-h IVNDFD	0.35	0.60	0.34

<sup>a</sup> Degrees of freedom were 774 for smooth brome grass, 279 for cocksfoot, and 728 for reed canarygrass.

<sup>b</sup> NDF, neutral detergent fibre; PCA, *p*-coumarate; IVNDFD, in vitro NDF digestibility.

Table 3

Summary statistics (mean, standard deviation, minimum, and maximum) for clone means of neutral detergent fibre ( $\text{g kg}^{-1}$  DM) and phenolic acid components of the cell wall ( $\text{g kg}^{-1}$  NDF)

Variable <sup>a</sup>	Smooth brome grass				Cocksfoot				Reed canarygrass			
	Mean	S.D.	Min.	Max.	Mean	S.D.	Min.	Max.	Mean	S.D.	Min.	Max.
NDF	548	14	494	587	608	4	596	621	600	9	517	629
Klason lignin	147	4	132	158	174	7	147	191	153	8	120	175
Esterified ferulate	4.40	0.21	3.55	5.00	4.66	0.24	3.76	5.51	6.10	0.25	5.40	7.02
Etherified ferulate	4.49	0.27	3.56	5.35	3.02	0.28	2.20	3.69	2.69	0.28	1.69	3.48
Esterified PCA	2.17	0.19	1.56	2.70	2.75	0.18	2.15	3.30	3.91	0.47	2.22	5.35
24-h IVNDFD	0.566	0.016	0.525	0.633	0.631	0.024	0.564	0.707	0.621	0.021	0.555	0.685
96-h IVNDFD	0.788	0.025	0.716	0.889	0.808	0.041	0.690	0.926	0.802	0.024	0.740	0.877

Means are over four harvest dates. Number of clones were 775 for smooth brome grass, 280 for cocksfoot, and 729 for reed canarygrass.

<sup>a</sup> NDF, neutral detergent fibre; PCA, *p*-coumarate; IVNDFD, in vitro NDF digestibility.

Table 4

Standardized partial least squares regression coefficients and their standard errors for the effects of neutral detergent fibre (NDF), Klason lignin, and ferulates on 24- or 96- in vitro neutral detergent fibre digestibility (IVNDFD)

Variable	Smooth bromegrass	Cocksfoot	Reed canarygrass
24-h IVNDFD			
NDF	$-0.847 \pm 0.017^{***}$	$-0.523 \pm 0.067^{***}$	$-0.474 \pm 0.050^{***}$
Klason lignin	$-0.265 \pm 0.019^{***}$	$-0.268 \pm 0.052^{***}$	$-0.124 \pm 0.044^{**}$
Esterified ferulate	$-0.026 \pm 0.018$	$0.019 \pm 0.053$	$-0.063 \pm 0.046$
Etherified ferulate	$-0.016 \pm 0.021$	$-0.312 \pm 0.082^{***}$	$-0.144 \pm 0.044^{**}$
Residual	0.385	0.500	0.783
96-h IVNDFD			
NDF	$0.014 \pm 0.013$	$-0.258 \pm 0.020^{***}$	$-0.153 \pm 0.022^{***}$
Klason lignin	$-0.596 \pm 0.015^{***}$	$-0.668 \pm 0.016^{***}$	$-0.505 \pm 0.020^{***}$
Esterified ferulate	$0.051 \pm 0.014^{***}$	$0.059 \pm 0.016^{***}$	$0.084 \pm 0.020^{***}$
Etherified ferulate	$-0.537 \pm 0.016^{***}$	$-0.390 \pm 0.025^{***}$	$-0.338 \pm 0.019^{***}$
Residual	0.293	0.152	0.339

\*\* Effect significantly different from zero at  $P < 0.01$ .

\*\*\* Effect significantly different from zero at  $P < 0.001$ .

for the other two species. The effects of lignin on 96-h IVNDFD were two to four times greater than on 24-h IVNDFD. Residuals were relatively small, indicating that these cell-wall components explained a large proportion of the variability in 96-h IVNDFD. The concentration of NDF had a small negative effect for cocksfoot and reed canarygrass and esterified ferulate had a small positive effect for all three species, but most of these effects were smaller in magnitude than the residuals.

For smooth bromegrass, Klason lignin was not correlated with NDF, but etherified ferulate had a strong positive correlation with NDF (Fig. 1A). There was a moderate and positive correlation between Klason lignin and etherified ferulate ( $r = 0.41$ ,  $P < 0.001$ ) for smooth bromegrass. For cocksfoot, these relationships were similar to those observed for smooth bromegrass, despite a significantly reduced range of NDF values for cocksfoot (Fig. 1B). There was a low positive correlation between Klason lignin and etherified ferulate ( $r = 0.24$ ,  $P < 0.001$ ) for cocksfoot. For reed canarygrass, all three variables were positively correlated with each other (Fig. 1C). Among the three grasses, the correlation of Klason lignin with ferulate ethers was greatest for reed canarygrass ( $r = 0.65$ ,  $P < 0.001$ ; Fig. 1C).

#### 4. Discussion

The moderate to high intraclass correlation coefficients indicated fairly consistent differences among clones and rankings across harvests. The moderate repeatability of clone performance across harvests was similar to observations made by Casler and Jung (1999). The moderate to high repeatability across harvests can be partially attributed to lack of variation in leaf:stem ratio, because these samples consisted entirely of leaf tissue (Casler, 1999a,b). Because leaves and stems differ considerably in lignin and NDF for these species, whole-plant samples may possess clonal variability in leaf:stem ratio, confounding true differences in lignin, NDF or phenolic acids.

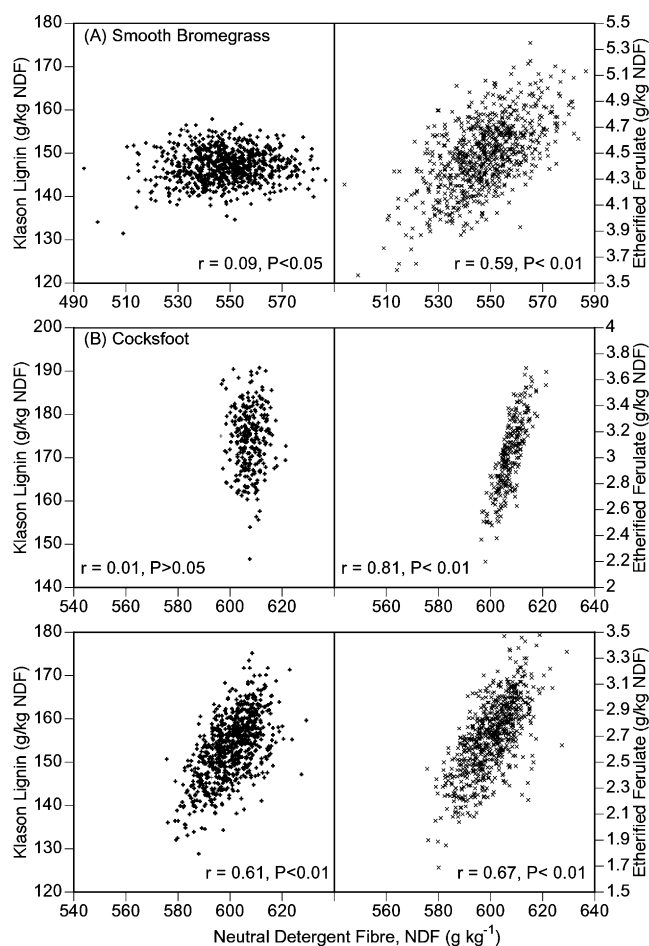


Fig. 1. Relationships of Klason lignin or etherified ferulate to neutral detergent fibre (NDF) concentration for: (A) 775 smooth bromegrass clones, (B) 280 cocksfoot clones, and (C) 729 reed canarygrass clones.

Our initial work on lignin and phenolic acids was based on smooth bromegrass, demonstrating considerable variability for several phenolic components of leaf tissue (Casler and Jung, 1999; Jung and Casler, 1991). However, both cocksfoot and reed canarygrass have considerably more variability for Klason lignin concentration than smooth bromegrass (Table 3), broadening the scope of this study compared to prior work and increasing the range of inference with regard to lignin concentration.

In vitro digestibility of cool-season-grass leaf tissue appears to be regulated by different mechanisms at 24 and 96 h. At 24 h, 49–62% of the fibre fraction has disappeared, depending on clone and species. Digestion proceeds most rapidly in cell walls that are the least lignified and have the least secondary wall development (Akin and Amos, 1975; Akin and Burdick, 1981), i.e. those with the lowest lignin and NDF concentrations. Because the anatomical composition of leaf blades can vary considerably among clones (Ehlke and Casler, 1985), it



is likely that digestion by rumen microbes proceeds at a faster rate in clones with more highly digestible tissues, such as mesophyll, parenchyma, and chlorenchyma cells. As digestion proceeds to its full extent at 96 h, 69–92% of the fibre fraction has disappeared. The concentration of NDF becomes less important because a large portion of the NDF fraction has been degraded and only the most resistant cell walls remain, those that are the most highly lignified (Akin, 1989). Both lignin and ferulate cross-linking become important because the limiting factors at later stages of digestion are the covalent linkages between lignin and arabinoxylans by ferulates (Jung and Deetz, 1993). The relative consistency of these effects across three species that differ in NDF, lignin, and phenolic acid concentrations, indicates that this phenomenon probably occurs in most forage grass species.

An ultimate goal of this research is to select genotypes and create populations of plants that differ in lignin and ferulate cross-linking concentrations in such a way that independent statistical comparisons can be made of the effects of these two cell-wall components on livestock performance. To do this, we must identify clones with the desirable traits, make crosses or polycrosses to produce seed populations, conduct further testing of those populations, then initiate feeding or grazing trials with those seed populations that are consistent with their parental selection criteria. Casler and Jung (1999) demonstrated the feasibility of identifying and selecting clones with orthogonal differences in Klason lignin and etherified ferulate of smooth brome grass, but the number of clones was too small to result in biologically meaningful progress.

The correlation structures for these grasses will make it difficult, but not impossible, to select orthogonal groups of clones with divergent lignin or ferulate cross-linking. For reed canarygrass, a large divergence in Klason lignin, with constant etherified ferulate, can only be achieved by severely restricting the range of possible values for lignin, limiting the potential range of inference. The results were similar for clones divergent in etherified ferulate with constant Klason lignin. For smooth brome grass and cocksfoot, the relatively low correlations between Klason lignin and etherified ferulate indicated that it should be possible to select divergent plants without restricting the range of values for the other trait.

However, the greater problem will be to avoid confounding differences in etherified ferulate with NDF, which were positively and strongly correlated within all three species. Selection of clones with divergent etherified ferulate, but with similar NDF, will severely restrict the potential divergence in etherified ferulate and, due to the severe reductions in selection differential, may be impossible within the limits of statistical precision. Divergent selection for etherified ferulate, ignoring NDF, could lead to significant confounding between these two variables, resulting in an inability to distinguish between two important factors each with potential negative effects on livestock performance. Because Klason lignin and NDF were not correlated for smooth brome grass and cocksfoot, this does not present a problem for divergent lignin selections. For reed canarygrass, the strong and positive correlation between Klason lignin and NDF will similarly make it impossible to identify orthogonal differences in lignin without severely restricting the range of both variables. The relatively low range of values for Klason lignin of smooth brome grass and NDF for cocksfoot may be partially responsible for the lack of correlation observed between Klason lignin and NDF in these two species. However, the low range of NDF values for cocksfoot did not prevent occurrence of a significant positive correlation between etherified ferulate and NDF.

## 5. Conclusions

Concentrations of NDF, Klason lignin, and ferulate ethers were all negatively correlated with IVNDFD in all three perennial grasses examined. The impact of ferulate cross-linking was more pronounced for potentially digestible NDF (96-h incubations) than the rapidly digestible NDF fraction (24-h incubations). However, the results indicate some serious potential problems associated with etherified ferulic acid as a selection criterion to improve digestibility of perennial grasses. For all three species, low etherified ferulic acid was strongly associated with low NDF. Because the correlations were not perfect, plants can be selected for low etherified ferulic acid within constant-NDF classes, resulting in no expected change in NDF concentration. However, this selection protocol will necessarily reduce selection differentials and genetic gains for reduced etherified ferulic acid, slowing the progress that can be achieved. For smooth brome grass and cocksfoot, Klason lignin concentration is clearly a better selection criterion than etherified ferulic acid, because low-lignin plants can be identified largely independent of NDF concentration. For these two species, Klason lignin may be an effective substitute for IVNDFD as a selection criterion when rumen fluid from a fistulated cow is not readily available. However, for reed canarygrass, Klason lignin is a problematic selection criterion, due to its strong positive relationship with NDF. Therefore, in reed canarygrass direct selection for IVNDFD will be required rather than selection on the basis of either Klason lignin or etherified ferulate.

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